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Multiplex PCR for detecting EHEC infections

The present invention originates from the diagnostic area of the detection of hemorrhagic diarrheal diseases.

Enterohemorrhagic E. coli pathogens (EHEC) are dangerous pathogens of diarrheal diseases which may be transmitted both by foodstuffs and by smear infections. Enterohemorrhagic E. coli organisms are able to form highly potent cytotoxins. These are proteins which have great similarity with the Shiga toxin of Shigella dysenteriae type 1 and are therefore called Shiga toxin 1 and Shiga toxin 2. The genes coding therefor for the respective subunits A and B are referred to as stxA1 computer accession number M19473) and stxB1 (computer accession number M19473) and stxA2 (computer accession number X07865) and stxB2 computer accession number X07865). Pathogenic E. coli strains may contain either one of the two Shiga toxin genes or else both. In addition, the pathogens may have further associated virulence factors such as EHEC intimin, EHEC hemolysin, EHEC catalase, EHEC serine protease and EHEC enterotoxin.

The diagnostic detection of EHEC is, because of the transmission routes and of the low infectious dose of about 10^2-10^3 organisms, important not only for those with acute disease but also in order to identify possible excretors or find other sources of infection. In the state of the art, EHEC infections are detected by microbiological means using

Thomas et al., Eur. J. Clin. Microbiol. Infect. Dis. 13, pp. 1074-1076, 1994). However, these infections were not detectable by prior art molecular methods for detecting human-pathogenic EHEC infections (Pierard et al., J. Clin. Microbiol. 36, pp. 3317-3322, 1998). Since corresponding immunological tests have only limited specificity, elaborate microbiological enrichment methods and subsequent molecular sequence analyses were carried out. It was thus possible to identify as enterohemorrhagic E. coli strains such pathogens which were previously known only as Shiga toxin-producing swine-pathogenic organisms (Pierard et al., Lancet 338, p. 762, 1991; Thomas et al., Eur. J. Clin. Microbiol. Infect. Dis. 13, pp. 1074-1076, 1994). The sequences of the Shiga toxin genes of these swine-pathogenic isolates differ distinctly from those of human-pathogenic strains and are referred to as $stx2_e$ (Weinstein et al., J. Bacteriol. 170, pp. 4223-4230, 1988; Franke et al., Journal of Clinical Microbiology Vol. 33 No. 12, pp. 3174-3178, 1995).

Thus, to date there is no simple immunological or molecular biological method which can be used to detect the responsible pathogens of all enterohemorrhagic diarrheal diseases in humans.

It is therefore an object of the present invention to provide a method with which both human-pathogenic and swine-pathogenic EHEC pathogens can be identified by a single detection reaction.

This object is achieved by a nucleic acid

amplification reaction for the detection of clinically relevant EHEC infections, with which it is possible simultaneously to identify stxA1 and stxA2 sequences which are derived both from human-pathogenic and from swine-pathogenic pathogens.

The invention thus relates to a method with which, in a multiplex amplification reaction for the detection of clinically relevant EHEC infections, both stxA1 and stxA2 sequences are amplified, and which is characterized in particular by amplification both of human-pathogenic stxA2 isoforms and of swine-pathogenic $stxA2_e$ isoforms. In this connection, the term "multiplex amplification reaction" refers to PCR methods in which at least two different primer pairs are used, one primer pair being used to amplify stx1 sequences and a second primer pair being used to amplify stx2 sequences. The term "swine pathogen" is used within the scope of this application for pathogens which have a Shiga toxin gene $stx2_e$ (Weinstein et al., J. Bacteriol. 170, pp. 4223-4230, 1988), computer accession number: M21534) and primarily cause edema disease in swine, but may also lead to diarrheal diseases and extraintestinal disease manifestations in humans.

Primers which have proved particularly suitable for carrying out the method of the invention have a length of 17-25 nucleotides, whose sequences is either identical to a sequence as shown in SEQ ID No. 1-4, whose sequences represent continuous part-sequences of one of the sequences

measurable fluorescence signal being emitted with suitable excitation. It is thus possible on the basis of the data obtained to determine quantitatively the amount of target nucleic acid originally employed.

In another, preferred embodiment, the multiplex amplification products are detected after completion of the amplification reaction, in which case, after hybridization of the FRET pair onto the target nucleic acid to be detected, the temperature is increased continuously in a melting curve analysis. At the same time, the emitted fluorescence is measured as a function of the temperature and, in this way, a melting temperature at which the FRET pair employed no longer hybridizes onto the sequence to be detected is determined. If there are mismatches between the FRET pair employed and the amplification product, the melting point is significantly depressed. It is possible in this way to identify with one FRET pair different target nucleic acids whose sequences differ from one another slightly through one or a few point mutations.

This principle is employed according to the invention in a multiplex amplification reaction for detecting EHEC infections, in which there is use of an internal standard which differs from the stxA1 or stxA2 wild-type sequence (computer accession number X07865) only in one or two point mutations. It is thus possible to distinguish amplified target nucleic acid and amplified internal standard from one another with the aid of a melting curve analysis.

experiments for cloned stxA2 DNA from human-pathogenic strains. Melting temperatures of about 63°C were found for the DNA of the remaining 12 stxA2-containing human isolates and for the DNA from the three swine-pathogenic strains, which certainly contain the $stx2_e$ allele. It can be concluded from the identical Tm that the 12 human-pathogenic isolates are attributable to swine-pathogenic EHEC strains and presumably likewise contain the $stx2_e$ allele. This supposition was confirmed by sequence analysis of the PCR products from the corresponding 12 isolates.

Overall, this example shows that both human-pathogenic and swine-pathogenic EHEC pathogens can be identified with the aid of the method of the invention.

Example 5: Specificity - avoidance of false-positive results

Specificity tests were carried out on 32 stx-negative bacterial strains listed in table 1. For this purpose, DNA was extracted as in example 1 from appropriate overnight cultures. The isolated DNA was subsequently investigated as in example 2 for the presence of stxA1 and stxA2 using the SybrGreen mode. The result was always unambiguously negative. As inhibition control, the DNA was mixed with DNA of the stx1- and stsx2-positive E. coli strain EDL 933 in a parallel mixture and tested for stx1 and stx2 in the same run, unambiguously positive signals being obtained without exception.